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Pharmacological Evidence for the Involvement of Calcium Entry through TRPV1 Channels in Nifedipine-Induced $[Ca^{2+}]i$ Elevation in Gingival Fibroblasts*

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ABSTRACT

Background: Among anti-hypertension drugs, calcium (Ca^{2+}) antagonists cause gingival overgrowth as a side effect. We previously discovered that this side effect was due to elevation of the calcium concentration in the cytosol ($[Ca^{2+}]i$). Ca^{2+} entry through non-selective cation channels (NSCCs) and Ca^{2+} release from intracellular Ca^{2+} stores are involved in this $[Ca^{2+}]i$ elevation. Furthermore, we discovered that calcium-sensing receptors (CaSRs) participate in nifedipine-induced $[Ca^{2+}]i$ elevation. Transient receptor potential (TRP) channels have been identified as NSCCs. In the present study, we undertook experiments to determine if TRPV1 channels are present in gingival fibroblasts and to ascertain if nifedipine-activated NSCCs are TRPV1 channels. **Methods:** Normal human gingival fibroblast Gin-1 cells were used. The $[Ca^{2+}]i$ was measured using a video-imaging analysis system with the Ca^{2+}-sensitive fluorescent dye fura-2/AM. **Results:** The NSCC inhibitor SKF96365 significantly inhibited nifedipine-induced $[Ca^{2+}]i$ elevation. TRPV1 channel agonists such as capsaicin, olvanil and resiniferatoxin concentration-dependently elevated the $[Ca^{2+}]i$. The TRPV1 channel activator anandamide concentration-dependently increased the $[Ca^{2+}]i$. The TRPV1 channel antagonists capsazepine, AMG9810, iodoresiniferatoxin, ruthenium red, and SB366791 significantly inhibited nifedipine-induced $[Ca^{2+}]i$ elevation. **Conclusion:** These results suggest that Ca^{2+} entry through TRPV1 channels is involved in the nifedipine-induced $[Ca^{2+}]i$ elevation seen in gingival fibroblasts. We describe here a modified version of our "calcium trigger theory".

Keywords: Nifedipine; Gingival Overgrowth; Gingival Fibroblast; TRPV1 Channel

1. Introduction

Among calcium (Ca^{2+}) antagonists, anti-hypertension drugs cause gingival overgrowth as a side effect [1]. We previously discovered that this side effect is due to elevation of the calcium concentration in the cytosol ($[Ca^{2+}]i$) and advocated the "calcium trigger theory" as the developing mechanism [2]. This theory was based on results showing that Ca^{2+} antagonists enhance Ca^{2+} entry through non-selective cation channels (NSCCs) [3] and Ca^{2+} release from the intracellular Ca^{2+} stores (endoplasmic reticula) [4].

Furthermore, we discovered that calcium-sensing receptors (CaSRs) participate in nifedipine (typical Ca^{2+} antagonist)-induced $[Ca^{2+}]i$ elevation. This hypothesis was based upon results demonstrating that CaSR agonists (gentamicin, neomycin, spermine, and $LaCl_3$) elevated the $[Ca^{2+}]i$ and that the CaSR antagonist NPS2390 inhibited nifedipine-induced $[Ca^{2+}]i$ elevation [5]. It has been reported that signals from CaSR stimulation activate NSCCs [6] and Ca^{2+} release from Ca^{2+} stores [7].

Transient receptor potential (TRP) channels have been identified as NSCCs based upon their function [8-10]. In particular, there are several reports describing transient receptor potential vanilloid type-I (TRPV1) protein channels or capsaicin receptors as NSCCs [11-15] and that TRP channels are expressed in most tissues [16]. These include smooth muscles in the portal vein [11], oocytes [12], peripheral sensory neurons [13,14], and central neurons [17]. Inoue *et al.* [11] found that the NSCC inhibitor SKF96365 blocked the permeability of divalent cations, and stated that TRP channels could be Ca^{2+}-permeable, non-selective cation channels. Furthermore, Stelt and Marzo [17] claimed that the transient receptor potential vanilloid type I (TRPVI) protein is a non-selective cation channel that belongs to a large family of TRP ion channels. In the present study, we undertook

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experiments to determine if TRPV1 channels are present in gingival fibroblasts and whether nifedipine-activated NSCCs are TRPV1 channels.

2. Materials and Methods

2.1. Cell Culture

Normal human gingival fibroblast Gin-1 cells were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Cells were cultured for 3 - 6 days in Dulbecco's modified Eagle medium (Medium 41; Dainippon Pharmaceutical Co. Ltd.). Cells (5×10^3 per cm^2) were plated on fibronectin-coated glass cover slips adhered to a flexiperm disc (Greiner Bio-One GmbH, Göttingen, Germany). The medium was supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. The medium also contained antibiotics (50 U/ml penicillin and 50 μg/mL streptomycin; Sigma-Aldrich, St. Louis, MO, USA) and was changed every 2 - 3 days.

2.2. Measurement of the [Ca^{2+}]i

The [Ca^{2+}]i was measured with the Ca^{2+}-sensitive fluorescent dye fura-2/AM (Dojindo Laboratories, Kumamoto, Japan). Cells were kept in a buffer comprising 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES-NaOH (pH 7.4). They were loaded with the dye by incubation in 5 μM fura-2/AM for 45 min at 37˚C. Cells were then washed to remove excess fura-2/AM and then incubated in fresh buffer (without fura-2/AM) for 15 min after incubation to allow intracellular cleavage of the acetoxymethyl ester conjugate (and thus activation) of fura-2. Excitation light from a xenon lamp was passed through a filter (340 nm or 360 nm). The emission wavelength for analyses was 500 nm. Changes in the fluorescence intensity of fura-2 in cells were recorded with a video-imaging analysis system (FC-400, Furusawa Laboratory Appliance, Kawagoe, Japan). The [Ca^{2+}]i was determined as the ratio of the fluorescence stimulated by excitation at 340 nm or 360 nm compared with a standard calibration curve obtained using a Calcium Calibration Buffer Kit I (Molecular Probes, Eugene, OR, USA).

To minimise leakage of fura-2, cells were kept at 32˚C during fluorescence measurements using a bath temperature controller (DTC-100A; DIA Medical Systems, Kunitachi, Japan). Cells were soaked in a flexiperm chamber containing 0.5 ml of saline and perfused at 8.0 ml/min with a tubing pump system (Master flex 7524-10; Cole-Parmer Instrument Company, Barrington, IL, USA). Drugs at appropriate concentrations were added to the perfusate. The time of treatment with CaSR agonists was 30 s. To ensure that fura-2 fluorescence was maintained within the linear range (*i.e.*, did not become saturated), we selected for analyses cells with a basal [Ca^{2+}]i in the range 50 - 200 nM.

2.3. Chemicals

Tissue culture reagents were purchased from Gibco BRL (Rockville, MD, USA). Nifedipine, capsaicin, olvanil, resiniferatoxin, anandamide, capsazepine, AMG9810, ruthenium red, iodoresiniferatoxin, and SB366791 were purchased from Sigma-Aldrich. SKF96365 was obtained from Calbiochem (San Diego, CA, USA). All other chemicals were supplied by Nacalai Tesque (Kyoto, Japan). These chemicals were dissolved in dimethyl sulfoxide (Sigma-Aldrich) as stock solutions, and thereafter added to the perfusate.

2.4. Statistical Analyses

Data are the mean ± standard error of the mean (SEM) and the number of observations (N). Statistical analyses of the data were undertaken by the Student's two-sided paired *t*-test. Differences between mean values were considered significant if the probability of error (p) was less than 0.05.

3. Results

The anti-hypertension drug isradipine is a dihydropyridine derivative, just like nifedipine. Isradipine has been shown to be antagonised by NSCC inhibitors such as SKF96365, $GdCl_3$, $HgCl_2$, and flufenamic acid [3]. To confirm that NSCCs are involved in nifedipine-induced [Ca^{2+}]i elevation, the interaction of SKF96365 with nifedipine was examined. SKF96365 significantly inhibited the effect of nifedipine (nifedipine alone, 60.24 ± 5.38 nM; SKF96365+nifedipine: 32.36 ± 5.59 nM (N = 32, $p < 0.001$)).

The effects of agonists of TRPV1 channels (capsaicin, olvanil, and resiniferatoxin) on the [Ca^{2+}]i were examined to confirm the existence of TRPV1 channels in gingival fibroblasts. Capsaicin (1 - 10 μM, **Figures 1(a)** and **(b)**), olvanil (1 - 20 μM, **Figure 1(c)**) and resiniferatoxin (0.5 - 5.0 μM, **Figure 1(d)**) concentration-dependently elevated the [Ca^{2+}]i.

We investigated the effect of an activator of TRPV1 channels, anandamide, on the [Ca^{2+}]i. Anandamide (1 - 50 μM) concentration-dependently raised the [Ca^{2+}]i (**Figure 2**).

The effects of antagonists of TRPV1 channels (capsazepine, AMG9810, iodoresiniferatoxin, ruthenium red, and SB366791) were tested. Capsazepine (10 μM), AMG9810 (10 μM), iodoresiniferetoxin (5 μM), ruthenium red (50 μM), and SB366791 (10 μM) significantly inhibited nifedipine-induced elevation of the [Ca^{2+}]i (**Figure 3**).

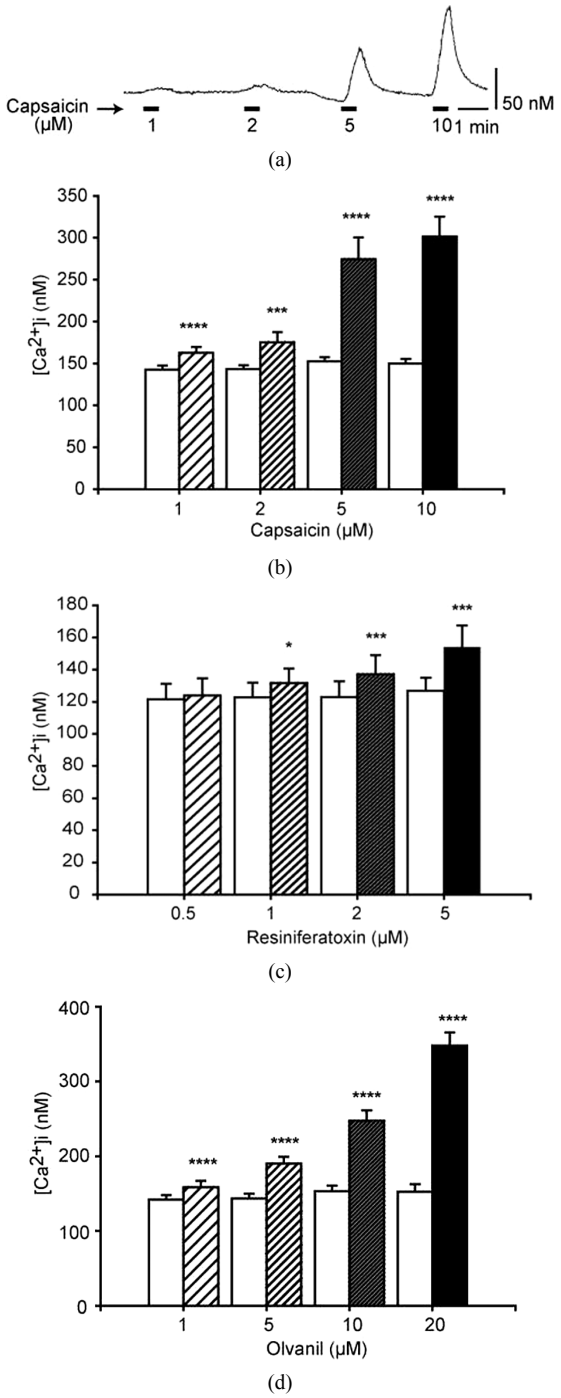

Figure 1. Elevation of the [Ca^{2+}]i by agonists of the TRPV1 channel. (a) The trace shows a representative time-course of the [Ca^{2+}]i in the case of capsaicin application; (b) [Ca^{2+}]i measurements were made in the presence (hatched or black bars) or absence (clear bars) of capsaicin (1, 2, 5, and 10 μM); (c) In the case of resiniferatoxin application (0.5, 1, 2, and 5 μM). (d) In the case of olvanil application (1, 5, 10, and 20 μM). N = 30 (capsaicin), 16 (resiniferatoxin), or 28 (olvanil). Data are mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.005$, and $^{****}p < 0.001$ compared with corresponding pretreatment values.

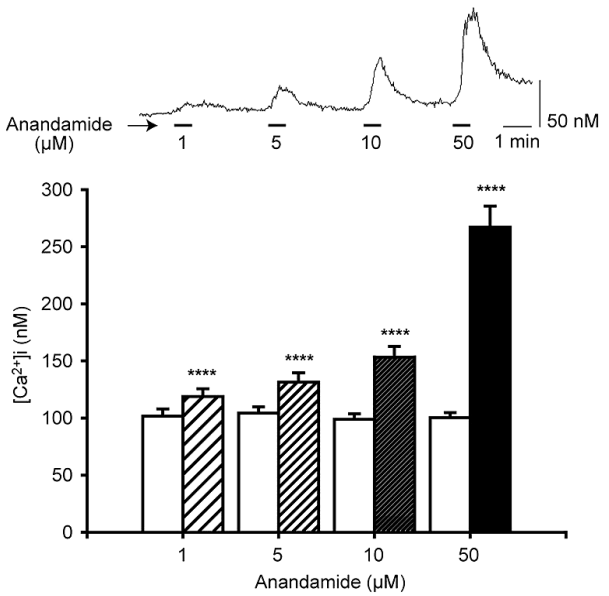

Figure 2. Elevation of the [Ca^{2+}]i by an activator of the TRPV1 channel, anandamide. The upper trace shows a representative time-course of the [Ca^{2+}]i in the case of anandamide application. [Ca^{2+}]i measurements were made in the presence (hatched and black bars) or absence (clear bars) of anandamide (1, 5, 10, and 50 μM). Data are mean ± SEM. N = 35. $^{****}p < 0.001$ compared with corresponding pretreatment values.

4. Discussion

We previously observed the involvement of CaSRs in nifedipine-induced elevation of the [Ca^{2+}]i [5]. There are reports that CaSR stimulation induces NSCC activation [18,19] and that NSCCs are TRPV1 channels [11-14, 17,20,21]. With respect to TRPV1 channels in non-excitable cells, Myrdal and Steyger [11] reported that there are more than 20 members of a newly described group of membrane proteins that act as receptors and ion channels: the TRP family. They are non-selective, calcium-permeant cation channels. Most of them are non-voltage-gated and are involved in calcium homeostasis (especially in non-electrically active cells). Thus, the present study was conducted to determine if TRPV1 channels are involved in nifedipine-induced [Ca^{2+}]i elevation in gingival fibroblasts.

The effect of the NSCC inhibitor SKF96365 on nifedipine-induced [Ca^{2+}]i elevation was investigated to confirm a relationship between the actions of Ca^{2+} antagonists and NSCCs in gingival fibroblasts. Nifedipine-induced [Ca^{2+}]i elevation was inhibited by SKF96365, confirming that Ca^{2+} entry though NSCCs is involved in nifedipine-induced elevation of the [Ca^{2+}]i.

To ensure the expression of TRPV1 channels in gingival fibroblasts, effects of agonists (capsaicin, olvanil, and resiniferatoxin) and an activator of TRPV1 channels

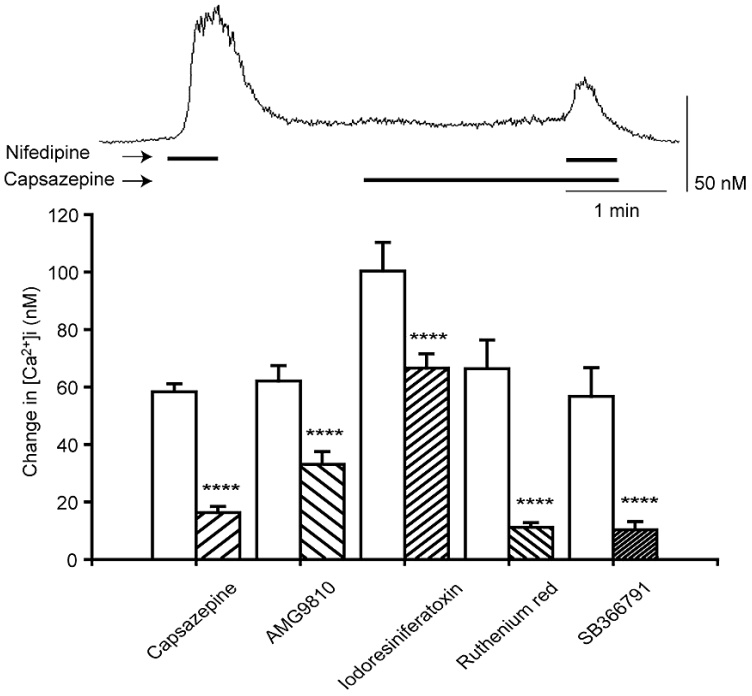

Figure 3. Inhibition of nifedipine-induced [Ca^{2+}]i elevation by antagonists of the TRPV1 channel. The upper trace shows a representative time-course of the [Ca^{2+}]i in the case of capsazepine application. [Ca^{2+}]i measurements were made in the presence (hatched or black bars) or absence (clear bars) of capsazepine (10 μM), AMG9810 (10 μM), iodoresiniferatoxin (5 μM), ruthenium red (50 μM), and SB366791 (10 μM). Data are mean ± SEM. N = 23 (capsazepine), 35 (AMG9810), 18 (iodoresiniferatoxin), 24 (ruthenium red), or 29 (SB366791). $^{****}p < 0.001$ compared with corresponding pretreatment values.

(anandamide) were tested. All elevated the [Ca^{2+}]i confirming, on a functional level, that TRPV1 channels were present in gingival fibroblasts.

The effects of antagonists of TRPV1 channels (capsazepine, AMG9810, iodo-resiniferatoxin, ruthenium red, and SB366791) on nifedipine-induced elevation of the [Ca^{2+}]i were examined to ascertain if nifedipine enhances Ca^{2+} entry through TRPV1 channels. All of the antagonists of TRPV1 channels significantly inhibited nifedipine-induced [Ca^{2+}]i elevation. These results suggested that Ca^{2+} entry through TRPV1 channels was involved in nifedipine-induced [Ca^{2+}]i elevation in gingival fibroblasts.

From the results obtained from our previous studies [4,5], a "modified calcium trigger theory" regarding the mechanism of Ca^{2+} antagonist-induced gingival overgrowth could be postulated (**Figure 4**). The mechanism of [Ca^{2+}]i elevation involved three main steps. First, Ca^{2+} antagonists directly act on a CaSR-linked Gq protein, which activates phospholipase Cβ to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Second, IP_3 and DAG induce the activation of IP_3 receptors in Ca^{2+} stores and activation of protein kinase C, respectively. Third, the [Ca^{2+}]i is elevated by Ca^{2+} release from Ca^{2+} stores and by Ca^{2+} entry through TRPV1 channels. In addition, sustained elevation of [Ca^{2+}]i, which is nec-

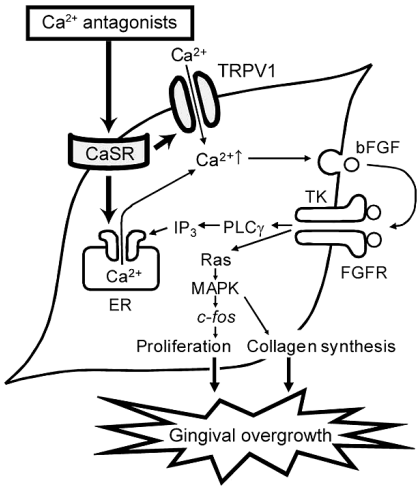

Figure 4. Mechanism of Ca^{2+} antagonist-induced gingival overgrowth: the "modified calcium trigger theory". At first, Ca^{2+} antagonists elevate the [Ca^{2+}]i by directly stimulating CaSRs, which activate TRPV1 channels and enhance Ca^{2+} release from endoplasmic reticula. These actions finally become an indispensable "trigger" of gingival overgrowth. CaSR: calcium-sensing receptor; TRPV1: transient receptor potential V1 channel; bFGF: basic fibroblast growth factor; FGFR: basic fibroblast growth factor receptor; TK: tyrosine kinase; PLCγ: phospholipase Cγ: IP3: inositol 1,4,5-triphosphate; ER: endoplasmic reticulum; and MAPK: mitogen-activated protein kinase.

essary in cell proliferation [22], is caused by positive feedback related to the secretion of basic fibroblast growth factor and activation of tyrosine kinase.

5. Conflicts of Interests

The authors declare that they have no conflict of interests to disclose.

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